Original Paper

Neonatology
formerly Biology of the Neonate

Neonatology 2009;96:50–60
DOI: 10.1159/000201739

Received: October 24, 2007
Accepted: June 30, 2008
Published online: February 10, 2009

Quantification of *Hox* and *Surfactant Protein-B* Transcription during Murine Lung Development

David G. Grier[a] Alexander Thompson[b] Terence R.J. Lappin[b]
Henry L. Halliday[a, c]

[a]Regional Neonatal Unit, Royal Maternity Hospital, and [b]Haematology Research Group, Centre for Cancer Research and Cell Biology, and [c]Department of Child Health, Queen's University, Belfast, UK

Key Words
Hox genes · Lung development · Gene expression · *Surfactant protein-B* · Real-time quantitative polymerase chain reaction

Abstract
Background: Genetic processes underlying fetal lung development and maturation are incompletely understood. Better knowledge of these processes would provide insights into the causes of lung malformations and prevention of respiratory distress syndrome and the potential adverse effects of glucocorticoids. *Hox* genes are involved in the lung branching morphogenesis and maturation of respiratory epithelium, but their expression pattern remains to be defined. ***Objectives:*** We hypothesized that genes involved in lung branching would be downregulated during early development, whereas those involved in maturation would be unchanged or upregulated. ***Methods:*** TaqMan real-time primers and probes were designed for all 39 murine *Hox* genes, and the murine *SP-B* gene and transcription profiles of these genes were obtained from whole lungs isolated at e14.5, e16.5, e18.5, e19.5 and postnatal days 1 and 20. ***Results:*** *Hox* genes in clusters A and B, specifically those between paralog groups 3 and 7, were the most represented, with *Hoxa4* and *Hoxa5* being the most highly transcribed. A wave of reduced transcription in 16 *Hox* genes, coincident with increased *SP-B* transcription, was observed with advancing gestation. Consistently high transcription of *Hoxa5* from e14.5 to postnatal day 20 may indicate that sustained transcription is required for normal lung maturation. When e15.5 lungs were cultured with dexamethasone, *Hoxb6*, *Hoxb7* and *Hoxb8* levels were significantly upregulated, creating the potential for modulation of diverse downstream target genes. ***Conclusions:*** Improved understanding of the genetic processes underlying lung development afforded by our Q-PCR platform may allow development of more specific methods for inducing fetal lung maturation.

Introduction

In recent years, the use of antenatal glucocorticoids and postnatal exogenous surfactant has decreased the limits of viability to about 23 weeks' gestation. Current limits of viability of preterm neonates are determined by maturity of lung structural and functional development. Antenatal glucocorticoid administration is effective in reducing the incidence and severity of respiratory distress syndrome (RDS) by inducing structural and functional changes in the fetal lung [1]. Surfactant protein-B

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1661–7800/09/0961–0050$26.00/0

Accessible online at:
www.karger.com/neo

Dr. David G. Grier
Regional Neonatal Unit, Royal Maternity Hospital
Grosvenor Road
Belfast BT12 6BB (UK)
Tel. +44 28 9063 3829, Fax +44 28 9023 6203, E-Mail grier@doctors.org.uk

(SP-B) is a marker of fetal lung maturation, and deficiency causes RDS. One of the actions of antenatal glucocorticoids is to upregulate *SP-B*, but they may also alter the expression of other genes in many developing organs leading to potentially adverse effects. Novel, targeted methods to accelerate lung development will depend on improved understanding of the underlying molecular mechanisms involved in the control and regulation of lung development, which at present remain largely unknown [2].

Lung development is dependent upon the coordinated expression of a large number of genes in a manner tightly controlled both in time and space [3, 4]. Early in embryogenesis, gradients of transcription factors define developmental axes giving embryonic cells positional information. Homeobox genes are master regulatory genes involved in embryo pattern formation, organogenesis, cell differentiation and hematopoiesis [5]. Mammalian homeobox genes can be divided into class I, also known as *Hox* genes which occur in clusters, and class II or divergent which are found throughout the genome. In mammals, 39 *Hox* genes are arranged in 13 paralog groups in 4 clusters (A–D) with the low-numbered genes (e.g. *Hoxa1*) at the 3′ end of each cluster and the higher numbered (e.g. *Hoxa13*) at the 5′ end. The order of *Hox* genes within each cluster affects their pattern of expression, with 3′ genes expressed earlier in the developing embryo than 5′ genes (temporal colinearity) and 3′ genes expressed in rostral structures with 5′ genes expressed in more caudal structures (spatial colinearity). *Hox* genes are highly evolutionarily conserved with the result that there is a high degree of homology between human and murine *Hox* gene sequences [6, 7].

The importance of *Hox* genes in human lung development has been demonstrated by their abnormal expression in several congenital lung abnormalities. *Hoxb5* is necessary for normal airway branching during development and is overexpressed in bronchopulmonary sequestration [8] and in congenital cystic adenomatoid malformation [9]. The etiology of these disorders is largely unknown but each represents failure of areas of primordial lung tissue to follow normal patterns of morphogenesis. Altered patterns of *Hox* gene transcription have also been demonstrated in several acquired lung disorders, including emphysema and primary pulmonary hypertension [10] and some lung carcinomas [11].

Murine models suggest a role for *Hoxb5* in branching morphogenesis. In lung explants (e11.5) treated with *Hoxb5* antisense oligonucleotides, there was a foreshortening and reduction of the branches arising from the mainstem bronchi [12]. This supports a previous finding that all-*trans* retinoic acid, known to upregulate a number of *Hox* genes in explanted lungs including *Hoxb5*, caused elongation of the primary branches [13]. All-*trans* retinoic acid and altered *Hox* transcription may be the cause of abnormalities in lung branching morphology associated with retinoid excess or deficit [12]. Groenman et al. [3] have reviewed the relationships of retinoids, *Hox* gene regulation and abnormal lung development.

There is also strong evidence of the importance of the paralog *Hoxa5* for structural development of the respiratory system and regulation of pulmonary surfactant production. It has previously been reported that *Hoxa5* transcription is either constant or decreases slightly during lung development from e13.5 to d2 [13, 14]. Mice homozygous for a loss-of-function mutation of *Hoxa5* develop to full term but die in the early neonatal period [15]. These *Hoxa5*-deficient mice have tracheal occlusion and reduced expression of surfactant proteins A, B and C with pathology similar to surfactant-deficient RDS in preterm human neonates [15].

Previous studies using nonspecific and nonquantitative degenerate primers demonstrated the predominance of transcripts from clusters A and B in both mice and humans [10, 13, 16]. Quantitative and precise measurement of the transcription profiles of all 39 *Hox* genes is essential to critically evaluate the role of this gene network in lung development and maturation. We hypothesized that *Hox* gene transcription in lungs would follow the pattern of spatial colinearity seen in other developmental models. We further hypothesized that genes such as *Hoxb5*, suggested by explant studies to be involved in the lung branching morphogenesis, would be downregulated during early development, whereas those involved in the maturation of respiratory epithelium would be unchanged or upregulated. To test these hypotheses, we designed and validated a highly specific and sensitive TaqMan™ quantitative-polymerase chain reaction (Q-PCR) platform to measure all 39 *Hox* genes simultaneously along with *SP-B*.

Materials and Methods

Design of Real-Time Q-PCR Primers and Probes

TaqMan (Applera, Foster City, Calif., USA) real-time primers and probes were designed for all 39 murine *Hox* genes and the *SP-B* gene (table 1) using GenBank published sequences and Primer Express™ software (Applera). Q-PCR reactions, analysis and validation of the target amplicons were carried out as previously described [17]. Total RNAs isolated from all major adult and fetal

Table 1. Design of TaqMan primers and probes

Gene	Accession No.	Site	Forward primer	TaqMan™ probe (5′ FAM, 3′ TAMRA)
Hoxa1	NM 010449	Exon 1	CCTTGGCAGTGGCGACTCT	CGAGCTTACCCCTCTGACCATGGGAT
Hoxa2	NM 010451	Exon 1	TCGCTGAGTGCCTGACATCT	CCCCTGTCGCTGATACATTTCAAAGTTCA
Hoxa3	Y11717	Exon 1	CAATGGGTTCGCTTACAATGC	CAGCCATACGCGCCGTCCG
Hoxa4	S67058	Exon 2	CCGGAGAATGAAGTGGAAGAAA	CACAAACTTCCCAACACCAAGATGCGA
Hoxa5	NM 010453	Exon 1	TAGTTCCGTGAGCGAACAATTC	CTCGGCGAGCATGCACTCCG
Hoxa6	AF247663	Exon 1	CCTATTTTGTGAATCCCACTTTCC	CCTGCCCAGCGGCCAGGA
Hoxa7	NM 010455	Exon 1	ACGCGCTTTTTAGCAAATATACG	CTTCTCTCTTCCAAAATGCCGAGCCG
Hoxa9	NM 010456	Exon 1	CCGAACACCCCGACTTCA	TGCAGCTTCCAGTCCAAGGCGG
Hoxa10	NM 008263	Exon 1	CACAGGCCACTTCGTGTTCTT	TGCGCAGAACATCAAAGAAGAGAGCTCC
Hoxa11	NM 010450	Exon 1	AGATTTCTCCAGCCTCCCTTCTT	CCCCAGACCCCGTCTTCGCG
Hoxa13	NM 008264	Exon 2	CCTCCCCACCTCTGGAAGTC	TCTCCCATCCTTCAGACGCCAGCTC
Hoxb1	NM 008266	Cross intron	ACTCTCACTCCCCGGACCTT	AGAGAAACCCACCTAAGACAGCGAAGGTGTC
Hoxb2	M34004*	Exon 1	GCTCGCCGAGTGTCTGACTT	CCCCGCTGTCTTGGAGACATTTCAAA
Hoxb3	U02278	Exon 1	TACCAGCGCTCAGCGTGTT	TGCAGTCCCTGGGCAACGCC
Hoxb4	M36654	Exon 1	ACTCAAACTATGTCGACCCCAAGT	CACAGAGCGATTACCTACCCAGCGACC
Hoxb5	NM 008268	Cross intron	GGGCAGACTCCACAGATATTCC	ATGAGGAAGCTTCACATCAGCCACGATATGA
Hoxb6	J03782	Cross intron	TTCCTATTTCGTGAACTCCACCTT	AGCGGGCAGGAGTCCTTCCTGG
Hoxb7	X06762	Cross intron	TCTAAATATCCAGCCGCAAGTTC	TTTCGCTCCAGGAGCCTTCCCC
Hoxb8	X13721	Exon 1	AACTCACTGTTCTCCAAATACAAAACC	AGTCCCTGCGCCCCAATTATTATGACTG
Hoxb9	S66855*	Cross intron	TGTCCATTTCTGGGACGCTTA	ACGCCGAGCACCTGGACTTCCC
Hoxb13	NM 008267	Exon 1	CGCTGATGCCAACTGTCAAC	CCCCCCTGGATCTGCCAGGC
Hoxc4	NM 013553	Cross intron	AACCCATAGTCTACCCTTGGATGA	ATTCACGTTAGCACGGTGAACCCCAATTATAA
Hoxc5	NM 008271	Cross intron	ACCCGTGGATGACCAAACTG	ATGAGCCACGAGACGGATGGCAA
Hoxc6	S74185	Exon 1	ACGTCGCCCTCAATTCCA	CCTATGATCCAGTGAGGCATTTCTCGACC
Hoxc8	X07439	Cross intron	TCTCCCAGCCTCATGTTTCC	ATGAGACCCCACGCTCCTGGGC
Hoxc9	NM 008272	Exon 1	TGTAGCGATTTTCCGTCCTGTAG	AGCCGGCTGTATTCAGTACGTCGTGG
Hoxc10	XM 128109	Exon 1	GGGCCAAGACCGCAGACT	AAGCTAAAGGTAAGGCCGTAGGAGATAAAGGCA
Hoxc11	XM 111600	Exon 1	GCGGCCGACGAGCTTAT	CACCGGGAGTGCCTGCCTCCT
Hoxc12	U04839*	Exon 1	TCTCCTGAATCCTGGGTTTGTG	TGGTGAATATCCACACAGGAGACACCTTCTACTT
Hoxc13	U04838*	Exon 1	GCCACCCTGGGCTATGG	CTACGGCTGCCGCCTGTCGC
Hoxd1	NM 010467	Cross intron	CGCCCACAGCACTTTCG	AACGCCCCCAAGAAAAGCAAACTGTC
Hoxd3	NM 010468	Cross intron	AAAGAATCCCGACAGAACTCCAA	TGTGCCACTTCAGGAGAGAACTGTGAGGA
Hoxd4	NM 010469	Cross intron	GCTGTGGTCTACCCTTGGATGA	CACGTGAATTCGGTGAACCCCAACTACA
Hoxd8	X56561*	Exon 1	GGGAGCCCGCGAAGTT	ACGGATACGATAACTTACAGAGACAGCCGATTTT
Hoxd9	NM 013555	Cross intron	CGGGCTGCTCGCTGAA	TGACCCAAACAACCCTGCAGCGA
Hoxd10	NM 013554	Cross intron	CTGCCTGGCTGAGGTTTCC	AGAAGGAAAGCAAAGAGGAAATCAAGTCTGATACTCC
Hoxd11	X71422	Cross intron	GGAACGCGAGTTTTTCTTTAATGT	CAACTCTCTCGGATGCTCAACCTCACTGAC
Hoxd12	NM 008274	Exon 1	CCTGTGCCTCCAGCTTCAA	AGACACCAAAGGCCCGCTCAACTTG
Hoxd13	NM 008275	Cross intron	AGGTGTACTGTGCCAAGGATCAG	ATCATCCTTTCCAGGAGATGTGGCTTTAAACC
SP-B	NM 147779	Exon 8	AGCGCTACACAGTTCTCCTGCTA	TGGCCTTGTCCTCCGATGTTCCACT

* Indicates those designed using genomic sequence as mRNA sequence was not available or was incomplete.

tissues including whole fetuses were pooled, reverse-transcribed using Moloney leukemia virus native reverse transcriptase (Invitrogen, Paisley, UK) and used for the validation of the primer probe sets. Amplicons generated using the forward and reverse primers were cloned into TOPO-TA (Invitrogen) or pGEM-T Easy (Promega, Southampton, UK) and plasmid DNA was prepared using the Qiagen® miniprep kit (Qiagen Ltd., Crawley, UK), all according to the manufacturer's protocols.

Standard Curve Generation and Definition of Copy Number

The sequence-validated minipreps were each diluted 1:10 with ddH_2O, and plasmid DNA concentration was determined spectrophotometrically by absorption at 260 nm (A_{260} value) using an Ultraspec 4300 pro spectrophotometer (Amersham Biosciences, Uppsala, Sweden). An A_{260} value of 0.1 or higher was deemed satisfactory and in terms of plasmid purity, an A_{260}:A_{280} of 1.7–2.0 was accepted. A stock solution of 10^9 plasmid copies

Reverse primer	Standard curves		
	slope	intercept with y-axis	correlation coefficient
GCGCAGGATTGGAAAGTTGT	–3.162	37.826	0.999
AAAGCGTCGAGGTCTTGATTG	–3.159	37.238	0.999
AGGCAGGTCGATGGTACTCAAC	–3.508	38.157	0.999
GCCGAGGCAGTGTTGGAA	–3.058	37.892	0.999
GCTGAGATCCATGCCATTGTAG	–3.536	38.610	0.999
CAGCTGGCCCAAGAAGGA	–3.908	41.921	0.992
GGGTGCAAAGGAGCAAGAAG	–3.382	37.409	0.999
TTCCACGAGGCACCAAACA	–3.438	37.769	0.999
TTGTCCGCAGCATCGTAGAG	–3.387	39.229	0.998
TGGAGGAGTAGGAGTATGTCATTGG	–3.296	38.068	0.998
TAAGGCACGCGCTTCTTTCT	–3.464	39.490	0.998
CTGGCGCGTGGTGAAGTT	–3.494	38.860	0.996
AATGTCGACTCCTTGATTGATGAA	–3.265	38.594	0.999
TGCCATTGAGCTCCTTGCT	–3.422	38.053	0.999
GGCTGGAAGCCGCTCTCT	–3.555	39.711	0.996
GGGTCAGGTAGCGATTGAAGTG	–3.479	40.023	0.998
CCGCATAGCCAGACGAGTAGA	–3.432	38.756	0.996
CAAAGGCGCAAGAAGTTTGTT	–3.278	37.195	0.998
TTGCGAAGGGTGCTGGAA	–3.401	39.737	0.997
GAACACCGGCGCTTTGG	–3.403	37.759	1.000
GACAAGGGTGGCACTGCTTT	–3.228	36.841	0.995
CGGTTGTAATGAAACTCTTTCTCTAATTC	–3.769	42.527	0.998
AGGGTCTGGTAGCGCGTGTA	–3.427	37.836	0.999
CTGAGCTACGGCTGCTCCAT	–3.437	37.627	0.998
GTCTGATACCGGCTGTAAGTTTGTC	–3.775	40.368	0.998
CCGTAAGGGTGATAGACCACAGA	–3.289	37.903	0.998
GCCAATTTCCTGTGGTGTTTTC	–3.398	39.201	0.998
TTTTTCATGAGGATCTCAGTGACTGT	–3.514	38.529	0.998
CCTGACGCGCGGAAGTT	–3.186	37.565	0.996
TTCTGCTGCAGGTTCACGTT	–3.493	38.209	0.999
GGGCTTGTGGCTCCATATTC	–	–	–
CACCAGCTGAGCACTCGTGTAC	–3.462	39.308	0.999
AATGAAATTCCTTTTCCAGTTCTAGGA	–3.489	40.183	0.998
CACTGGACGATTTACAGTCAGGAT	–3.353	38.190	0.997
TCCAGCTCTAGCGTCTGGTATTT	–3.520	40.317	0.998
AGCGTTTGGTGCTTGGTGTAA	–3.515	40.941	0.994
TCCTGCGATTCTGGAACCA	–3.176	37.467	0.997
GCCACTTGCACTGCCATGT	–3.313	37.118	0.998
TGGTGTAAGGCACCCTTTTCTT	–3.521	39.576	0.995
GGGCCCATGGCATCCT	–3.253	38.363	0.998

per μl was produced. Plasmids were linearized with *Not1* restriction enzyme digestion (New England Biolabs, Beverly, Mass., USA) and diluted to produce a solution with final concentration of 10^8 template copies per μl. Serial dilutions of plasmid in ddH_2O were used to provide a range of copy numbers from 10^1 to 10^7 per μl.

Q-PCR analysis using the serially diluted plasmids as templates was performed in triplicate (table 1). All standard curves, correlation coefficients, gradient and intercept values were generated using the ABI 7700 sequence detection system and associated software (Applera). The correlation coefficient value indicates the reproducibility of the TaqMan system over several orders of magnitude. The y-intercept value indicates the level of sensitivity of the system for each target, and the gradient reflects the robustness of the kinetics of the PCR over a range of template concentrations. Standard curve generated copy number values

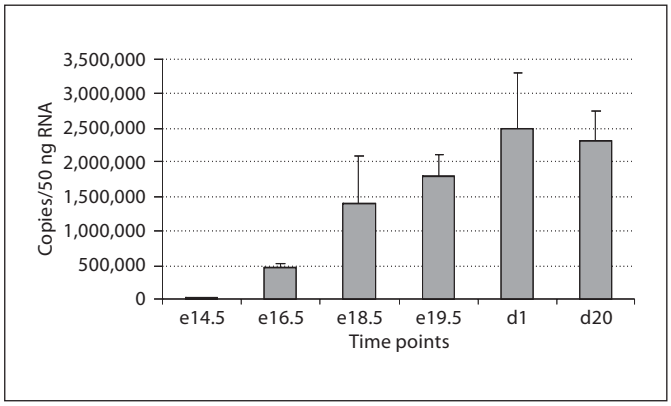

Fig. 1. Copies of *SP-B* mRNA detected in 50 ng total RNA obtained from murine lungs at 6 time points: e14.5, e16.5, e18.5, e19.5 and postnatal days 1 and 20. The transcription of *SP-B* increased over 21-fold between e14.5 and e16.5 and then increased almost 3-fold at e18.5 before plateauing.

were calculated using the threshold cycle (C_T) values obtained from Q-PCR of murine lung cDNA.

Murine Fetal Lungs

C57BL/6J recipient mice were obtained from Harland Laboratories (Bicester, UK). Timed matings were achieved by placing female mice with males overnight. Evidence of a copulation plug the following morning denoted e0.5. The care and use of all animals in this study was approved by the Research Ethics Committee of Queen's University, Belfast, with animal handling and welfare regulated by the Animals (Scientific Procedures) Act (1986) of the UK.

The mice were killed by carbon dioxide at the noted time (n = 3 dams per time point). The fetuses were removed by hysterotomy, assessed for expected gestational stage and placed into ice-cold Hanks' buffered salt solution (HBSS; Invitrogen). Since litter number affects the size and development of embryos, only dams with litter sizes 7–8 were analyzed. Each fetus was dissected under sterile conditions using 23- and 25-gauge hypodermic needles. A linear incision was made along both sides of the thorax and transversely across the root of the neck, and the thoracic cage was reflected and removed by an inferior incision above the diaphragm. The trachea and descending aorta were identified and transected. The intrathoracic organs were placed in ice-cold HBSS and the heart, thymus and extrapulmonary airways were removed by fine dissection under a light microscope (Carl Zeiss, Oberkochen, Germany). The lungs were obtained from 4 fetuses from each dam. The right and left lungs from the 4 fetuses were pooled, macerated using a razor blade and passed through a 25-gauge needle before being dissolved in 1 ml Trizol™ (Invitrogen). One male and one female pup were obtained from each of 3 litters at 4–6 h of age (d1) and of three 20-day-old litters (d20). The lungs were pooled from each litter and dissolved in 1 ml Trizol.

Short-Term Culture of Primary Lungs

To determine the effects of dexamethasone on *Hox* gene transcription, lungs from e15.5 fetal mice were obtained as described above and cultured in 200 μl serum-free Waymouth medium (Gibco, Paisley, UK) with 1% penicillin and streptomycin at 37°C in 5% CO_2 and 21% O_2. The media contained either 10^{-7} M dexamethasone (Aldrich, Poole, UK) or vehicle (0.04% ethanol). After 8 h, 1 day or 3 days in culture, the lungs were washed with HBSS and dissolved in 1 ml Trizol. Lungs cultured for 3 days had media replaced daily.

Q-PCR

Total RNA was extracted from Trizol, quantified by absorbance at 260 nm, and the quality was assessed by agarose gel electrophoresis (data not shown). The RNA was subsequently treated with DNase1 (Invitrogen) and reverse transcribed using Moloney leukemia virus native reverse transcriptase as previously described [15]. Q-PCR was performed on 50 ng equivalents of cDNA from total RNA in triplicate for each *Hox* gene and *SP-B* using the ABI 7700 sequence detection system. Predeveloped TaqMan Assay Reagents were used to measure 18S ribosomal RNA. The C_T for each Q-PCR reaction was recorded in triplicate, the mean was calculated and corrected for 18S ribosomal RNA transcription levels.

The copy numbers of mRNA for each gene per 50 ng of total RNA were calculated using the standard curves. Regression analysis and analysis of variance were performed on the corrected C_T values using SPSS for Windows v11.0 (SPSS Inc., Chicago, Ill., USA).

Results

Changes in Transcription with Lung Maturation

SP-B mRNA in lungs increased from low levels at e14.5 to high levels at e18.5, which were maintained in the postnatal period (fig. 1). The transcription profile of all 39 *Hox* genes was quantified in lungs at each of the 6 time points during fetal and postnatal development (table 2; fig. 2). The highest transcribed *Hox* genes were those in clusters A and B, particularly those in paralog groups 3–6. *Hoxa4* was the most highly transcribed *Hox* gene at e14.5 (139,053 copies/50 ng RNA), while *Hoxa5* was the most highly transcribed in postnatal lungs (48,755 copies/50 ng RNA in newborn lungs). *SP-B* gene transcription was analyzed as a positive indicator of lung maturation, and levels increased from e14.5 (20,985 copies/50 ng RNA) to e18.5 (1,370,241 copies/50 ng RNA) and persisted after the postnatal period as expected.

In the A cluster genes, transcription of *Hoxa1* was relatively low at all time points (2,035 and 1,466 copies/50 ng RNA in e14.5 and newborn lungs, respectively), whereas the transcription of *Hoxa2* was slightly higher (7,868 and 3,661 copies/50 ng RNA in e14.5 and newborn

Table 2. Mean threshold cycles for *Hox* genes and *SP-B* during lung development

	e14.5	e16.5	e18.5	e19.5	d1	d20	e14.5 to d1	
							Corr.	p
Hoxa1	27.44 ± 0.55	28.40 ± 0.46	28.78 ± 0.93	28.60 ± 0.38	27.96 ± 0.79	28.19 ± 0.77	0.34	0.2091
Hoxa2	25.05 ± 0.76	25.70 ± 0.22	26.70 ± 1.16	26.23 ± 0.45	26.00 ± 0.26	26.27 ± 0.51	0.53	0.0428*
Hoxa3	23.51 ± 0.97	24.08 ± 0.57	24.91 ± 1.34	24.52 ± 0.65	24.34 ± 0.68	24.33 ± 0.88	0.41	0.1284
Hoxa4	22.20 ± 0.39	23.25 ± 0.54	25.22 ± 1.49	23.86 ± 0.20	24.57 ± 0.78	23.91 ± 0.51	0.69	0.0048*
Hoxa5	21.66 ± 1.05	21.83 ± 0.73	22.92 ± 1.36	22.45 ± 0.53	22.12 ± 0.63	22.39 ± 0.60	0.32	0.2462
Hoxa6	25.22 ± 0.50	26.37 ± 0.59	27.73 ± 1.04	27.09 ± 0.50	27.03 ± 0.45	27.84 ± 0.64	0.70	0.0035*
Hoxa7	26.04 ± 1.01	26.35 ± 1.32	26.66 ± 2.10	26.84 ± 0.81	26.65 ± 0.89	25.66 ± 1.46	0.23	0.4020
Hoxa9	29.66 ± 1.01	30.16 ± 4.33	29.92 ± 4.51	31.67 ± 1.50	30.58 ± 1.55	29.60 ± 3.47	0.19	0.5084
Hoxa10	31.18 ± 1.00	33.90 ± 0.17	33.08 ± 1.13	33.75 ± 0.58	31.67 ± 1.29	32.25 ± 1.74	0.19	0.5024
Hoxa11	29.51 ± 0.66	31.43 ± 2.03	30.90 ± 1.54	31.92 ± 1.25	30.23 ± 1.54	30.45 ± 1.53	0.24	0.3801
Hoxa13	36.53 ± 3.01	38.10 ± 4.59	39.45 ± 3.41	37.26 ± 3.66	31.77 ± 1.99	38.02 ± 0.91	–0.28	0.3076
Hoxb1	31.75 ± 1.06	35.91 ± 0.78	36.12 ± 1.50	35.91 ± 0.84	33.39 ± 0.69	33.20 ± 1.76	0.37	0.1745
Hoxb2	27.20 ± 1.05	28.74 ± 1.36	29.40 ± 2.30	29.30 ± 1.03	29.31 ± 0.82	30.51 ± 1.13	0.52	0.0454*
Hoxb3	23.15 ± 0.49	24.54 ± 0.17	25.95 ± 0.11	25.18 ± 0.30	25.49 ± 0.47	26.08 ± 0.51	0.83	0.0001*
Hoxb4	24.38 ± 1.25	25.51 ± 0.79	27.05 ± 0.94	25.68 ± 0.92	26.62 ± 0.68	26.61 ± 0.39	0.61	0.0158*
Hoxb5	24.70 ± 1.58	25.86 ± 1.00	26.65 ± 1.98	26.99 ± 0.71	27.03 ± 0.92	27.18 ± 0.82	0.62	0.0141*
Hoxb6	26.09 ± 1.14	27.96 ± 0.47	29.67 ± 1.08	29.24 ± 0.61	29.28 ± 0.68	29.23 ± 0.86	0.81	0.0003*
Hoxb7	27.23 ± 0.58	28.19 ± 0.10	29.30 ± 0.42	28.74 ± 0.31	28.54 ± 0.34	28.60 ± 0.66	0.70	0.0036*
Hoxb8	27.52 ± 1.26	29.49 ± 0.74	31.36 ± 1.55	31.53 ± 0.51	31.26 ± 0.59	32.18 ± 1.13	0.83	0.0001*
Hoxb9	29.37 ± 1.06	29.73 ± 0.50	32.31 ± 0.84	31.35 ± 1.26	31.02 ± 0.87	32.59 ± 2.49	0.61	0.0152*
Hoxb13	30.54 ± 0.70	35.11 ± 1.53	32.84 ± 1.84	34.65 ± 1.21	31.46 ± 1.30	32.16 ± 1.89	0.18	0.5323
Hoxc4	30.36 ± 1.78	31.37 ± 1.00	32.67 ± 1.68	32.32 ± 0.80	32.72 ± 0.73	32.98 ± 1.33	0.62	0.0133*
Hoxc5	29.84 ± 1.08	32.17 ± 1.47	33.04 ± 1.92	34.24 ± 2.46	33.51 ± 0.57	32.40 ± 1.34	0.70	0.0036*
Hoxc6	28.93 ± 1.35	30.04 ± 2.14	30.90 ± 1.68	31.11 ± 2.19	30.18 ± 1.35	30.87 ± 3.36	0.36	0.1895
Hoxc8	30.60 ± 2.79	32.86 ± 2.38	34.11 ± 3.29	34.96 ± 3.79	35.06 ± 3.71	36.00 ± 5.27	0.52	0.0465*
Hoxc9	30.81 ± 0.77	34.63 ± 1.08	33.15 ± 1.88	34.70 ± 1.86	32.28 ± 1.53	33.15 ± 2.31	0.30	0.2832
Hoxc10	31.73 ± 1.01	33.97 ± 1.13	33.20 ± 1.36	35.59 ± 0.80	35.03 ± 1.66	34.81 ± 1.14	0.68	0.0050*
Hoxc11	30.31 ± 1.45	34.77 ± 0.43	32.12 ± 3.08	33.94 ± 1.28	31.44 ± 2.18	32.62 ± 2.14	0.15	0.5986
Hoxc12	30.75 ± 0.60	34.98 ± 0.50	33.44 ± 1.04	35.03 ± 0.81	32.20 ± 1.34	32.74 ± 1.89	0.32	0.2375
Hoxc13	29.81 ± 0.95	33.89 ± 1.27	32.68 ± 1.88	33.45 ± 1.20	31.05 ± 1.45	31.85 ± 2.13	0.25	0.3746
Hoxd1	38.25 ± 3.03	38.92 ± 1.88	39.00 ± 1.74	38.78 ± 2.11	39.72 ± 0.49	39.85 ± 0.26	0.23	0.4173
Hoxd3	33.22 ± 1.19	34.57 ± 1.00	36.79 ± 0.77	34.60 ± 0.58	34.99 ± 0.17	35.38 ± 0.46	0.49	0.0618
Hoxd4	32.92 ± 1.30	34.74 ± 3.66	33.92 ± 4.26	35.55 ± 4.91	33.01 ± 0.68	32.73 ± 2.70	0.08	0.7807
Hoxd8	31.00 ± 0.87	30.80 ± 1.96	32.69 ± 1.69	29.67 ± 1.94	29.57 ± 0.38	30.68 ± 0.77	–0.25	0.3618
Hoxd9	35.14 ± 1.49	34.04 ± 2.09	34.76 ± 1.90	32.94 ± 2.70	32.98 ± 0.06	33.94 ± 1.43	–0.41	0.1272
Hoxd10	35.97 ± 1.38	36.23 ± 0.85	36.80 ± 1.18	36.87 ± 3.73	33.70 ± 0.51	34.84 ± 0.97	–0.21	0.4583
Hoxd11	31.47 ± 0.81	36.23 ± 0.07	35.39 ± 1.34	36.14 ± 1.27	32.30 ± 1.60	33.29 ± 1.83	0.23	0.4184
Hoxd12	30.93 ± 0.79	33.27 ± 0.83	32.88 ± 2.43	33.23 ± 2.88	31.47 ± 1.66	32.64 ± 2.44	0.15	0.5928
Hoxd13	35.43 ± 1.47	38.07 ± 3.00	39.77 ± 1.12	39.18 ± 1.16	39.06 ± 1.56	39.90 ± 0.64	0.63	0.0124*
SP-B	24.78 ± 1.49	19.97 ± 0.33	18.82 ± 1.35	18.06 ± 0.40	17.70 ± 0.81	17.71 ± 0.46	–0.86	0.2091*

Corr. = Pearson's correlation for C_T value to age from e14.5 to d1. * $p < 0.05$.

lungs, respectively). There was an increasing level of transcription from 3′ to 5′, reaching a maximal level of transcription in *Hoxa4* or *Hoxa5* depending on the time point. In e14.5 lungs, there were 139,053 copies of *Hoxa4* and 71,200 copies of *Hoxa5* mRNA/50 ng RNA, and in newborn lungs there were 25,296 copies of *Hoxa4* and 48,755 copies of *Hoxa5* mRNA/50 ng RNA. *Hox* genes from *Hoxa6* to *Hoxa9* had progressively lower levels of transcription with *Hoxa10* to *Hoxa13* having very low levels of transcription; 225, 363 and 257 copies of *Hoxa10*, *Hoxa11* and *Hoxa13* mRNA/50 ng RNA in newborn lungs.

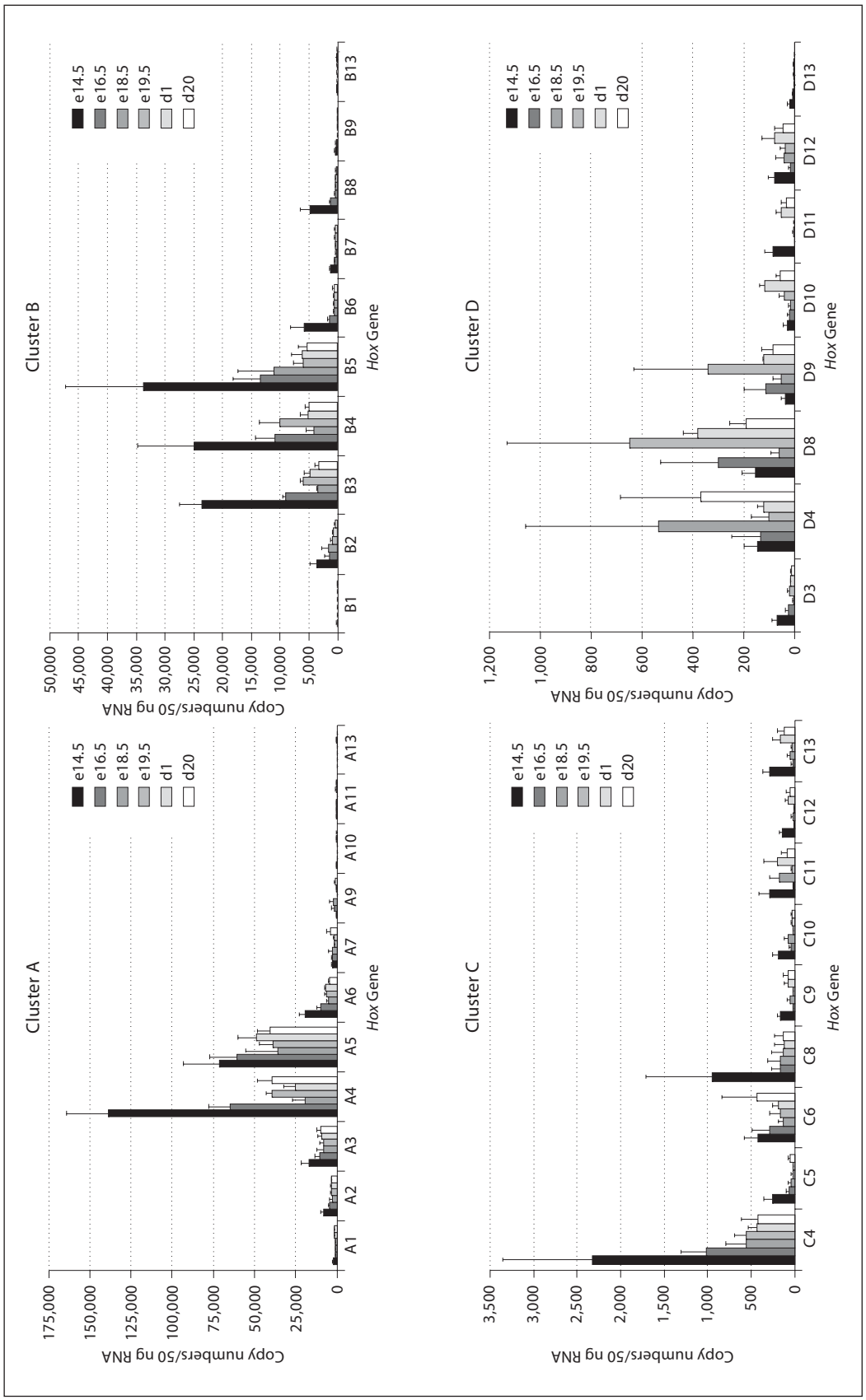

Fig. 2. Copies of each *Hox* mRNA (clusters A–D) detected in 50 ng total RNA, obtained from murine lungs at 6 time points. A cluster genes have the highest transcription, followed by B and C cluster genes with D genes the lowest. This is reflected by the changing vertical scales.

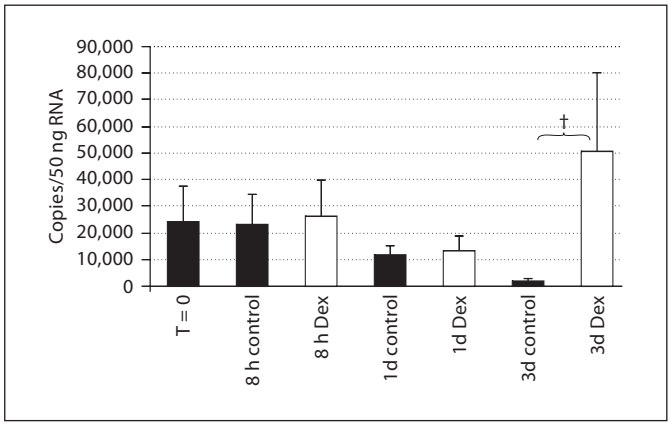

Fig. 3. *SP-B* mRNA copies detected in 50 ng total RNA, obtained from e15.5 murine lungs cultured with or without 10^{-7} M dexamethasone (Dex) for up to 3 days. 8 h, 1 and 3 days. † $p < 0.01$.

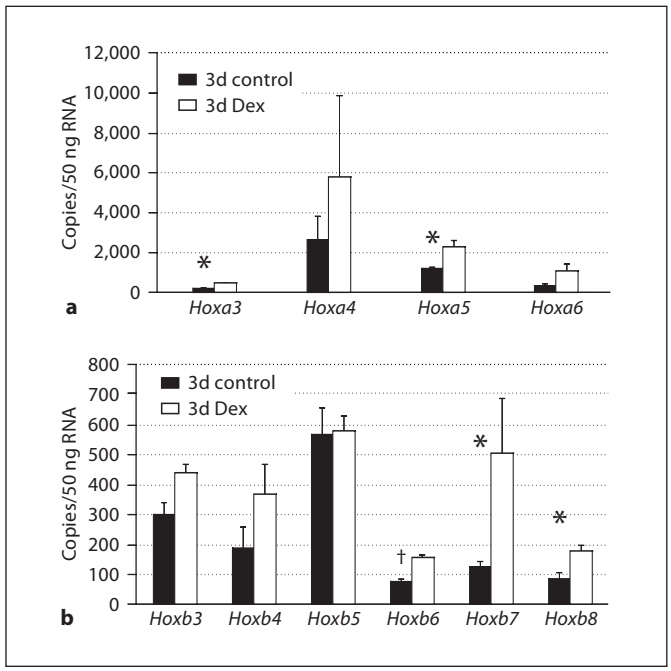

Fig. 4. a The effect of 3 days of 10^{-7} M dexamethasone (Dex) on A cluster *Hox* transcription in cultured e15.5 murine lungs. **b** The effect of 3 days of 10^{-7} M Dex on B cluster *Hox* transcription in cultured e15.5 murine lungs. * $p < 0.05$, † $p < 0.01$.

A similar pattern of transcription was found in the B cluster. Very low transcription levels of *Hoxb1* were evident at all time points (128 and 39 copies/50 ng RNA in e14.5 and newborn lungs) with progressively higher transcription levels of *Hoxb2–Hoxb3* (770 and 4,860 copies/50 ng RNA in newborn lungs), with maximal transcription of *Hoxb4* or *Hoxb5* (5,167 and 6,083 copies/50 ng RNA in newborn lungs). As with the A cluster genes, there were progressively lower transcription levels of 5′ genes, from *Hoxb6* (615 copies/50 ng RNA in newborn lungs) to *Hoxb13* (62 copies/50 ng RNA in newborn lungs). An exception to this pattern was *Hoxb8*, which had a higher level of transcription than *Hoxb7* in antenatal but not postnatal lungs. In e14.5 lungs, there were 1,151 copies of *Hoxb7* mRNA and 4,788 copies of *Hoxb8* mRNA/50 ng RNA, whereas in newborn lungs, there were 447 copies of *Hoxb7* mRNA and 328 copies of *Hoxb8* mRNA/50 ng RNA.

Of the C cluster genes, *Hoxc4* had the highest level of transcription at almost all time points (427 copies/50 ng RNA in newborn lungs), with *Hoxc6* and *Hoxc8* being the next highest transcribed (183 and 119 copies/50 ng RNA in newborn lungs). The remainder of the C cluster genes had lower levels of transcription, which were similar to each other. The D cluster genes had a low level of transcription with *Hoxd4*, *Hoxd8* and *Hoxd9* having the highest transcription in this cluster (121, 382 and 122 copies/50 ng RNA in newborn lungs).

A wave of falling transcription to less than 50% of e14.5 levels at e19.5 was detected for 33 of the *Hox* genes, and this was statistically significant for 16 of them. *Hoxa4* transcription decreased to 19% of e14.5 levels in postnatal day 1 lungs, *Hoxa6* transcription fell to 34% of e14.5 levels. *Hoxa5* transcription did not change significantly. *Hoxb4*, *Hoxb5* and *Hoxb6* transcription all declined during lung maturation to 21, 18 and 11% of e14.5 transcription, and *Hoxc5* transcription decreased to 7%. The transcription pattern of *Hoxa5* therefore stood out as different from other *Hox* genes of the same paralog group as well as genes adjacent within the same cluster.

Cultured Lungs

The transcription of *SP-B* in the e15.5 lungs (pseudoglandular phase) decreased significantly after 24 h in culture and declined further following an additional 3 days in control media. However, 3 days in culture with 10^{-7} M dexamethasone resulted in a significant increase in *SP-B* transcription compared to time-matched controls (fig. 3). The transcription levels of the 11 most highly transcribed *Hox* genes were measured, i.e. *Hoxa3* to *Hoxa6*, *Hoxb3* to *Hoxb8* and *Hoxc8*. In the absence of dexamethasone, a rapid (8-hour) and prolonged (3-day) downregulation of *Hox* gene transcription was observed (table 3). There was increased transcription of 5 *Hox* genes in explants after 3 days of culture with dexamethasone compared to controls (fig. 4). Dexamethasone caused a doubling of *Hoxa3, Hoxa5, Hoxb6* and *Hoxb8* transcription after 3 days of

Table 3. Mean threshold cycles for selected *Hox* genes and *SP-B* in explanted lungs

		T = 0	8 h		1 day		3 days	
			control	Dex	control	Dex	control	Dex
Hoxa3	C_T	23.32 ± 0.75	24.19 ± 0.81	24.15 ± 0.87	26.06 ± 0.22	26.07 ± 0.57	29.99 ± 0.32	28.76 ± 0.09
	p		0.9585		0.9905		0.0031*	
Hoxa4	C_T	22.72 ± 1.28	24.06 ± 1.43	24.24 ± 1.30	26.38 ± 1.07	25.95 ± 1.23	27.85 ± 1.48	27.05 ± 1.58
	p		0.8812		0.6702		0.5560	
Hoxa5	C_T	21.48 ± 0.69	22.40 ± 0.68	22.23 ± 0.64	24.72 ± 0.24	24.11 ± 0.28	27.72 ± 0.15	26.76 ± 0.37
	p		0.7717		0.0474*		0.0147*	
Hoxa6	C_T	25.88 ± 1.37	26.98 ± 1.04	26.63 ± 1.09	30.19 ± 0.65	29.42 ± 0.87	32.06 ± 0.70	30.24 ± 0.91
	p		0.7065		0.2901		0.0515	
Hoxb3	C_T	23.84 ± 0.59	24.79 ± 0.49	25.03 ± 0.24	26.94 ± 0.52	26.81 ± 0.17	29.63 ± 0.38	29.02 ± 0.18
	p		0.4826		0.7019		0.0673	
Hoxb4	C_T	25.75 ± 0.90	26.01 ± 0.62	26.32 ± 0.44	28.88 ± 0.89	28.41 ± 0.90	31.90 ± 1.26	30.76 ± 0.93
	p		0.5082		0.5594		0.2752	
Hoxb5	C_T	24.55 ± 0.27	25.11 ± 0.13	25.51 ± 0.21	27.09 ± 0.33	26.86 ± 0.10	30.48 ± 0.40	30.42 ± 0.21
	p		0.0557		0.2942		0.8279	
Hoxb6	C_T	26.72 ± 0.41	27.72 ± 0.27	28.16 ± 0.37	30.08 ± 0.61	29.83 ± 0.03	32.32 ± 0.29	31.22 ± 0.12
	p		0.1797		0.5104		0.0036*	
Hoxb7	C_T	26.63 ± 0.79	26.39 ± 0.69	26.46 ± 0.77	29.10 ± 0.30	28.03 ± 0.87	30.35 ± 0.38	28.50 ± 0.82
	p		0.9056		0.1162		0.0235*	
Hoxb8	C_T	27.41 ± 0.34	28.00 ± 0.29	28.45 ± 0.32	30.71 ± 0.34	29.95 ± 0.18	33.26 ± 0.60	32.09 ± 0.29
	p		0.1407		0.0265*		0.0373*	
Hoxc4	C_T	30.68 ± 0.48	30.18 ± 0.61	29.73 ± 0.66	31.82 ± 0.40	31.99 ± 0.40	34.89 ± 0.77	35.40 ± 0.50
	p		0.4337		0.6394		0.3911	
SP-B	C_T	24.50 ± 1.26	24.46 ± 1.08	24.31 ± 1.14	25.22 ± 0.67	25.19 ± 0.96	27.62 ± 0.67	23.54 ± 1.36
	p		0.8782		0.9666		0.0096*	

* $p < 0.05$.

culture (*Hoxa3* from 216 to 477 copies/50 ng RNA, *Hoxa5* from 1,208 to 2,294 copies, *Hoxb6* from 76 to 157 copies and *Hoxb8* from 85 to 180 copies). Doubling of *Hoxb7* transcription occurred after 1 day of dexamethasone treatment (713 copies/50 ng RNA in dexamethasone-treated compared to 300 copies in control) and by 3 days, the transcription in dexamethasone-treated lungs (507 copies/50 ng RNA) was 4 times that of controls (126 copies/50 ng RNA).

Discussion

The Q-PCR platform designed, validated and employed in this study enabled the measurement of gene transcription in a developmental lung model with a high degree of specificity and sensitivity. This is critical for the evaluation of gene expression profiles of highly related genes such as the *Hox* family. Specificity was attained by the rational design of primer-probe sets for each cDNA to be measured, and sensitivity over a dynamic range of 6 logs was provided by TaqMan chemistry. The generation of standard curves of the primer-probe sets enabled, for the first time, the quantification of transcription of each gene in terms of copy numbers based on plasmid number equivalents.

The prevalence of 3′ *Hox* genes of clusters A and B in murine lungs at all developmental time points is consistent with the pattern of expression in previous published studies [10, 13, 16]. The temporal decrease in transcription of a subset of *Hox* genes indicates possible roles for them in the regulation of early lung developmental processes such as branching morphogenesis. *Hoxa4*, in particular, is very highly transcribed in early lung development, but levels decline in later lung development. The maintenance of a high level of *Hoxa5* transcription during later lung development is consistent with its possible role in surfactant regulation [15].

The absence of transcription of either *Hoxb1* or *Hoxd1* in the lung can be explained by the expression of these

genes early in development in more caudal structures than the lungs. In situ hybridization using RNA probes for *Hoxb1* has previously demonstrated no transcription in the fetal mouse lung, whereas *Hoxb2*, *Hoxb3*, *Hoxb4* and *Hoxb5* were all detected in a region-specific fashion in the e9.5 to e12.5 developing lung [18].

Chinoy et al. [19] found that Hoxb5 protein, as measured by Western blotting and immunohistochemistry, decreased progressively in e14 mouse lungs cultured for 3–7 days. They concluded that the decline in Hoxb5 protein was similar to that seen during normal development in vivo and reflected maturation of the explanted lungs in vitro. Our study confirms and extends these findings by illustrating decreases in the mRNA of all 11 B cluster *Hox* genes including *Hoxb5*. *Hoxa5* mRNA levels, which remain constant during normal development in vivo, decrease during lung culture in vitro, suggesting exhaustion of a positive regulator of this gene ex vivo. This could be a general effect since a global fall in *Hox* transcription was observed in vitro. These findings also highlight the difficulty of reproducing in vivo developmental findings in culture models.

Surprisingly, we found no downregulation of *Hoxb5* mRNA following 3 days of culture with dexamethasone. It has previously been reported that protein levels of Hoxb5 are reduced following glucocorticoid-induced lung maturation [19]. It is possible that dexamethasone exerts its effects on *Hoxb5* at the posttranscriptional level, e.g. by reducing the translation efficiency or increasing protein degradation [1, 20]. In our study, the transcription of *Hoxb6*, *Hoxb7* and *Hoxb8* was increased by dexamethasone, indicating the complexity associated with hormone-induced *Hox* regulation. Glucocorticoids have multiple effects on the developing lung, including the induction of structural changes during alveolarization and increased expression of genes required for adaptation of the lung to air breathing [1]. The identification of this dexamethasone-regulated subset of genes provides a basis for potential novel strategies for lung maturation. Further experiments in vivo will be required to ascertain if the changes in *Hox* expression in cultured lungs will reflect the physiologic and pharmacologic events observed in utero.

Conclusion

A real-time PCR platform has been designed, validated and applied to quantify the transcription of the complete *Hox* network during murine lung development. A subset of developmentally regulated *Hox* genes has been identified both from in vivo and in vitro studies, suggesting that direct modulation of these master regulators will affect lung development and maturation in a positive way. More specific targeting of these processes, avoiding glucocorticoid-induced side effects, will have long-term benefit to the developing infant.

Grants

Dr. Grier is supported by a grant from the Research and Development Office of the Department of Health and Personal Social Services, Northern Ireland.

References

1 Grier DG, Halliday HL: Effects of glucocorticoids on fetal and neonatal lung development. Treat Respir Med 2004;3:295–306.
2 Burri PH: Structural aspects of postnatal lung development: alveolar formation and growth. Biol Neonate 2006;89:313–322.
3 Groenman F, Unger S, Post M: The molecular basis for abnormal lung development. Biol Neonate 2005;87:164–177.
4 Roth-Kleiner M, Post M: Genetic control of lung development. Biol Neonate 2003;84:83–88.
5 Mark M, Rijli FM, Chambon P: Homeobox genes in embryogenesis and pathogenesis. Pediatr Res 1997;42:421–429.
6 Zhang J, Nei M: Evolution of Antennapedia-class homeobox genes. Genetics 1996;142:295–303.
7 Hart CP, Fainsod A, Ruddle FH: Sequence analysis of the murine Hox-2.2, -2.3 and -2.4 homeoboxes: evolutionary and structural comparisons. Genomics 1987;1:182–195.
8 Volpe MV, Archavachotikul K, Bhan I, Lessin MS, Nielsen HC: Association of bronchopulmonary sequestration with expression of the homeobox protein Hoxb-5. J Pediatr Surg 2000;35:1817–1819.
9 Volpe MV, Pham L, Lessin M, Ralston SJ, Bhan I, Cutz E, Nielsen HC: Expression of Hoxb-5 during human lung development and in congenital lung malformations. Birth Defects Res Part A Clin Mol Teratol 2003;67:550–556.
10 Golpon HA, Geraci MW, Moore MD, Miller HL, Miller GJ, Tuder RM, Voelkel NF: HOX genes in human lung: altered expression in primary pulmonary hypertension and emphysema. Am J Pathol 2001;158:955–966.
11 Calvo R, West J, Franklin W, Erickson P, Bemis L, Li E, Helfrich B, Bunn P, Roche J, Brambilla E, Rosell R, Gemmill RM, Drabkin HA: Altered HOX and WNT7A expression in human lung cancer. Proc Natl Acad Sci USA 2000;97:12776–12781.
12 Volpe MV, Vosatka RJ, Nielsen HC: Hoxb-5 control of early airway formation during branching morphogenesis in the developing mouse lung. Biochim Biophys Acta 2000;1475:337–345.

13 Bogue CW, Gross I, Vasavada H, Dynia DW, Wilson CM, Jacobs HC: Identification of Hox genes in newborn lung and effects of gestational age and retinoic acid on their expression. Am J Physiol 1994;266:L448–L454.
14 Kim C, Nielsen HC: Hoxa-5 in mouse developing lung: cell-specific expression and retinoic acid regulation. Am J Physiol Lung Cell Mol Physiol 2000;279:L863–L871.
15 Aubin J, Lemieux M, Tremblay M, Berard J, Jeannotte L: Early postnatal lethality in Hoxa-5 mutant mice is attributable to respiratory tract defects. Dev Biol 1997;192:432–445.
16 Mollard R, Dziadek M: Homeobox genes from clusters A and B demonstrate characteristics of temporal colinearity and differential restrictions in spatial expression domains in the branching mouse lung. Int J Dev Biol 1997;41:655–666.
17 Thompson A, Quinn MF, Grimwade D, O'Neill CM, Ahmed MR, Grimes S, McMullin MF, Cotter F, Lappin TR: Global down-regulation of HOX gene expression in PML-RARα + acute promyelocytic leukemia identified by small-array real-time PCR. Blood 2003;101:1558–1565.
18 Bogue CW, Lou LJ, Vasavada H, Wilson CM, Jacobs HC: Expression of Hoxb genes in the developing mouse foregut and lung. Am J Respir Cell Mol Biol 1996;15:163–171.
19 Chinoy MR, Volpe MV, Cilley RE, Zgleszewski SE, Vosatka RJ, Martin A, Nielsen HC, Krummel TM: Growth factors and dexamethasone regulate Hoxb5 protein in cultured murine fetal lungs. Am J Physiol 1998;274:L610–L620.
20 Almawi WY, Abou-Jaoude MM, Li XC: Transcriptional and posttranscriptional mechanisms of glucocorticoid antiproliferative effects. Hematol Oncol 2002;20:17–32.